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TITLE: MOLECULAR BIOLOGY BASIS FOR THE RESPONSE OF POLY  
(ADP-RIB) POLYMERASE AND NAD METABOLISM TO DNA DAMAGE  
CAUSED BY MUSTARD ALKYLATING AGENTS

PRINCIPAL INVESTIGATOR: Mark E. Smulson, Ph.D.

CONTRACTING ORGANIZATION: Georgetown University  
37th and O Streets, N.E.  
Washington, DC 20057

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<p>During the midterm of this contract we have performed a variety of experiments to provide a strategy to modulate the nuclear enzyme poly(ADP-ribose) polymerase, in cultured keratinocytes. This enzyme modifies a variety of nuclear proteins utilizing NAD. DNA is required for the catalytic activity of the enzyme and the activity is dependent upon the presence of strand breaks in this DNA. It has been hypothesized that human skin exposed to mustards may develop blisters due to a generalized lowering of NAD in exposed skin cells. Furthermore, one mechanism may be the production of DNA strand breaks in skin cells by mustards which activate NAD depletion in these cells via the poly(ADP-ribose) polymerase reaction as described above.</p> <p>During the midterm we have cloned various regions of the cDNA for poly(ADP-ribose) polymerase into a series of retroviral expression vectors. We have shown that when keratinocytes are transfected with such expression vectors, increased amounts of poly(ADP-ribose) polymerase mRNA and protein are expressed. The project is currently exploring the use of a new retroviral system (M2) which offers several advantages over the retroviral expression system used earlier. Retroviral expression vectors should allow us to modulate the activity of this nuclear enzyme in a variety of skin cells in vitro and in vivo.</p> <p>See BLU's Key words</p>					
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## INTRODUCTION

The work of Papirmeister suggests that the marked depletion of NAD in order to generate poly(ADP-ribose) in response to DNA-strand-breaking agents occasioned by sulfur mustards depletes the cell of energy requirements and, ultimately by activating proteases, causes blisters in subcutaneous areas of skin (1). We are testing aspects of this hypothesis by quantitating the poly(ADP-ribose) metabolism in human skin cells. Understanding the basic mechanism of this process may allow reduction in pathology to the skin caused by such chemical warfare agents: The research directly addresses the basic mechanism underlying this effect and also provides newly updated data bases for assessment of identification of toxic effects caused by exposures to subsymptomatic doses of chemical warfare agents. It is quite clear that strand breaks in cellular DNA greatly stimulate the synthesis of poly (ADP-ribose) in nuclei of all eukaryotic cells including those of the skin. The substrate for the enzyme that synthesizes poly (ADP-ribose) is NAD, and, accordingly, when skin cells are exposed to chemical warfare agents causing strand breaks, NAD levels in these cells have been shown to drop dramatically. Papirmeister *et al.* (USAMRICD) have proposed that the above-noted depletion of NAD in skin cells will cause inhibition of glycolysis, and the resulting accumulation of common intermediates which stimulate the NADP-dependent hexomonophosphate shunt(1). This in turn leads to enhancement of protease synthesis and release from cells. These proteases could be responsible for development of subepidermal blisters. While partial validation of this biochemical hypothesis has been achieved, the studies underway in the current program will further substantiate this hypothesis by using both the recombinant DNA and immunological probes specific for poly(ADP-ribose) which are unique to our laboratory. The anticipated results obtained by the study will contribute to the protection of military personnel against a variety of chemical warfare agents, many of which cause DNA strand breaks and activation of the poly ADP-ribose system.

In the case of poly-ADP-ribosylation, many of the biological functions appear to be related to the stability of the chromatin adjacent to DNA strand breaks.

Thus the rationale for our project during the Early and Midterm period has been the hypothesis that nuclear poly-ADP-ribosylation is a key defense of the cell against DNA damaging agents such as alkylating agents, sulfur mustards and other agents to which the skin of military personnel may be exposed. Sulfur mustard is an alkylating agent that has cytotoxic, carcinogenic, and vesicant properties. Papirmeister *et al.* have presented a biochemical hypothesis explaining the formation of pathology in human skin exposed to mustard gas (1). This mechanism links the initiation of DNA damages induced by the mustard gas to local alterations of metabolism and subsequent development of blisters. It is clear that mustard gas alkylation ultimately leads to alkylation of purine bases in DNA that are ultimately repaired by endonucleases which in part make breaks in the phosphodiester bond of DNA. Our laboratory was one of the first to indicate that such strand breaks activate the chromosomal enzyme poly (ADP-ribose) polymerase. This enzyme utilizes NAD as a substrate and, at vesicating doses of mustard gas, depletes the cells of their NAD content. Papirmeister *et al.* have proposed that this depletion of NAD causes inhibition of glycolysis, and results in the stimulation of the NADP-dependent hexomonophosphate shunt.(1) Stimulation of the hexomonophosphate shunt has been associated with DNA damage and

enhancement of protease synthesis and release. Papirmeister concludes that these proteases could be responsible for development of subepidermal blisters--a characteristic feature of sulfur gas exposed to human skin. It is noteworthy that the participation of poly(ADP-ribose) polymerase activity in the mustard gas-induced NAD loss was substantiated by the observation of the prevention of this loss in the presence of inhibitors of the poly(ADP-ribose) polymerase enzyme (2).

Our study directly assesses the effects of sulfur mustard agents on the poly (ADP-ribose) content of the skin under these conditions by engineering skin cells with recombinant clones for the gene and cDNA for this enzyme.

During the early and mid-periods of this contract, we decided that the best available methodology to achieve both practical and very basic new information concerning poly(ADP-ribose) and mustards, in skin would be to stably integrate into skin cells extra copies of poly(ADP-ribose) polymerase cDNA or anti-cDNA (antisense mRNA). Such cells, once established and characterized, might be able to be maintained as a layer in nude mice. These cells will eventually allow us to:

- (1) Cause increased synthesis of the polymerase enzyme in skin tissue.
- (2) Cause decreased synthesis (via antisense) of the enzyme.
- (3) Alter the DNA repair, and survival capacity of engineered cells.

These new goals of the project were made possible because of recent progress in our laboratory in the cloning of the gene and cDNA for polymerase.

We have culminated a 2-year program on the sequencing of the cDNA for poly(ADP-ribose) polymerase as well as a formal study on the chromosome localization of the polymerase gene. The amino acid sequence obtained by my laboratory for poly(ADP-ribose) polymerase has been important for execution of the contract for a number of reasons; in particular, it allowed us to synthesize selected peptides to be used to produce more antibody to the protein. Additionally, it has allowed us to begin to understand the various functional domains (i.e., active site, DNA-binding domain, etc.) of this protein. Hence, in engineered keratinocytes, we plan to use this information in future years to engineer important regions of the enzyme into various retroviral vector so that we may be able to manipulate, in keratinocytes, the biological functions of poly(ADP-ribose) polymerase. Second, the chromosomal localization has allowed us to assign restriction sites to isolate the total active gene for the enzyme. The isolation of the gene may be very useful in future years in order to develop engineered keratinocytes and skin cells containing not only the cDNA sequences but also active gene sequences including introns.

Mulligan has developed a series of retroviral vectors (direct orientation -DO) that permit the simultaneous expression of an inserted protein-coding sequence and a dominant-acting selectable marker (i.e. neomycin resistance) (3,4). In these vectors, an internal SV40 or human metallothionein promoter sequence serves to drive the sequence of the bacterial neomycinphosphotransferase gene, whereas viral LTR sequences are utilized to promote expression of sequences. Two of these retroviral vectors are currently being used for this contract work (see below). Thus vector pZIP and vector pDOL are available with proper restriction sites for insertion of portions of both sense and antisense orientations of poly(ADP-ribose) polymerase in the lab. In these vectors the viral 5' splice site, normally used

in the biosynthesis of the subgenomic env-encoding mRNA, has been eliminated. These vectors yield high transient stable titers of virus after transfection of viral packaging cell lines.

Retrovirally-mediated gene transfer will be a potentially powerful means for transferring genes into animal cells, *in vivo* and *in vitro* into a wide host range of the viruses and an efficient mechanism for integration of the viral genome into the host cell DNA. The results of Mulligan's study suggest that the important features of the DOL vectors is the independent expression of selectable marker sequences. Additionally, Mulligan's initial studies with these vectors indicated that the expression of the LTR-directed sequences was not inhibited by the proviral integration.

We were approved to alter the initial plans of the contract during the first Mid-period. Accordingly, we have five aims, all directed at using retroviral expression vectors to alter skin cell levels of poly ADP-ribosylation, and elucidation of mustard damage of cells:

- (1) Verification of Expression of Exogenous Sense and Anti-sense Orientations of Poly ADP-rib polymerase in Cultured Human keratinocytes.
- (2) Verification of the Transcription of Integrated Sequences in Human Keratinocytes.
- (3) Verification of Synthesis (or Lack of synthesis Due to Anti-sense) of Biologically and Enzymatically Active Poly (ADP-ribose) polymerase in Human Keratinocytes.
- (4) *In vitro* measurement of DNA repair due to mustard exposure of keratinocytes. Cultures of transfected keratinocytes showing either increased levels of polymerase or decreased activity for polymerase due to the integration and transcription of antisense sequences or due to the transcription of competing 5' BAM-BAM sequences (corresponding to the DNA binding domain of the protein )will be tested for their ability to repair and survive DNA strand breaks as elicited by mustards.
- (5) Keratinocytes that prove to have either reduced or elevated synthetic activity for poly(ADP-ribose) polymerase will be tested for their ability to form an epidermis when transplanted to an athymic mouse. Normal or transfected keratinocytes will be grown to confluence. The epithelial sheet will be detached and grafted to the subcutaneous tissue of the back of an athymic mouse with the basal side facing outward and hence completely surrounded by body fluids of the mouse. After 1 to 3 weeks, the grafts will be removed, snapped-frozen or fixed in formalin and processed for histological examination. According to Mulligan, the morphology of grafts established by transduced keratinocytes "was indistinguishable from that of grafts formed by normal keratinocytes."

We anticipate performing all the stated experimental procedures using immunological methods to ascertain the generation of poly(ADP-ribose) (or lack of generation due to inhibitory or antisense engineered sequences in skin cells) in the epidermis grafted to athymic mice with human skin biopsies in nude mice. The advantage of the molecular biology approach utilized in this project is that a more precise model of whether poly ADP-ribosylation mediates blistering in skin cells may be achieved by using the new methods.

## Initial Results During Midterm

Results obtained constructing Retroviral Vectors for Keratinocytes.

Toward our goal of determining the mechanism of poly(ADP-ribose) polymerase involvement in DNA damage caused by mustards, we have utilized during the first midperiod retroviral expression vectors in the human keratinocyte system. The basic strategy has been to modulate the level of polymerase in the cells and then assess their response to mustards, in an in vivo system, as transplanted skin in nude mice.

In order to achieve this, we have produced constructs of the human poly(ADP-ribose) polymerase cDNA in the pDOL plasmid vector in both sense and antisense orientation. The data obtained involved restriction enzyme digests of the DOL constructs obtained. They were hybridized by southern transfer to human polymerase cDNA. The obtained bands verify that we have successfully cloned: (1) the full length cDNA; (2) the 5' region of the cDNA; the 5' region of the cDNA in the pZIP vector. The data indicate that they were cloned in both the sense and antisense orientations.

The retroviral expression vector we have chosen to use is based on the murine virus retroviral genome linked to polyoma virus sequences. This unique hybrid vector incorporates characteristics of both viral genomes into a state-of-the-art vector for introducing cDNA sequences into a variety of mammalian cells.

The vector contains a psi sequence which allows packaging of the mRNA transcript into virus particles, but the vector sequences do not code for viral particle proteins. When DNA plasmid vector is transfected into a packaging cell line (psi-2)--a cell line which constitutively produces viral particles but no packageable viral genome--the vector, in its RNA form, is packaged and shed into the media as virus. This virus can then be collected and used to transduce mammalian cells.

This vector system has several advantages:

- (1) Retroviruses have high transduction efficiencies, allowing introduction of cDNA sequences into a large number of cells.
- (2) These retrovirus particles are amphotrophic and infect a wide variety of cell types from different mammalian species.
- 3 The viral genome contains a neomycin resistance gene which allows for antibiotic selection of transformants.
- (4) Since the retrovirus vector lacks viral particle sequences, it cannot reproduce in cells other than the packaging cell line.
- (5) Retroviral LTR sequences allow for efficient integration into the cellular genome.
- (6) A pBR322 origin of replication allows for propagation of the vector constructs as DNA plasmids in bacteria.

- (7) Polyoma protein sequences and polyoma origin of replication allow for transient transfections to express cDNA in mouse cells, for assessment of expressibility of constructions.

Very recently, Morgan and co-workers (4) have reported using the DOL vector to express the cDNA for human growth hormone in primary human keratinocytes, thus demonstrating the utility of the vector in the human system. We have made progress on the expression of poly(ADP-ribose) polymerase in keratinocytes, using the DOL vector.

Keratinocytes offer several advantages for this type of study:

1. Keratinocytes have already been shown to express human cDNA introduced through DOL retroviral transduction.
2. An SV40-immortalized keratinocyte cell line is routinely propagated in our laboratory.
3. Primary normal human foreskin keratinocytes are available to us through collaboration with investigators in the Department of Pediatrics at Georgetown University Hospital.
4. The differentiation state of human keratinocytes in culture can be controlled by modulation the  $Ca^{++}$  concentration in the medium
5. Terminally differentiated keratinocytes have been reported to lack poly(ADP-ribose) polymerase, thus providing a low background host cell for the vector.
6. The epithelial origin of keratinocytes makes them a useful model for potential studies of sulfur mustard mediated by a DNA strand break, since carcinomas (epithelial) predominate over sarcomas (fibroblastid) among human cancers.
7. Keratinocytes can be transplanted from tissue culture into nude mice and grown as skin tissue grafts.

Our first concern about the poly(ADP-ribose) polymerase cDNA-DOL construction that was cloned for this project during the last year was their functional integrity--that is, their ability to produce polymerase within cells. To prove this, we transiently transfected the constructions into mouse NIH/3T3 cells and assayed for polymerase activity on an "activity" gel. The sense construction of the polymerase cDNA was the only vector to show elevated activity levels. This confirms that the vector potentially may be able to induce active enzyme in mammalian cells.

Based upon data indicating functional integrity of the vectors, we have proceeded to package the vectors as infectious retroviral particles. We have transfected the plasmids into packaging cells and are currently selecting for high titer-producing clones. These clones will be used as a perpetual source of virus for all future experiments.

Concurrently with the work in viral production, we have used conventional DNA transfection procedures to establish keratinocytes cell lines with stably integrated vector-cDNA constructs. This has been achieved by transfecting the DOL constructs into keratinocytes and selecting G418-



resistant clones. A total of 18 clones have been produced and are currently being characterized in terms of polymerase activity. These clones provide us with useful alternatives, should the retroviral work become problematic. Also, they allow us an in vitro system to determine parameters for the in vivo work as proposed in the contract.

During the future year we shall have retroviral vectors to use in experimentation. This will allow us to move from immortalized keratinocytes into primary keratinocytes derived from human foreskin. These cells will be infected with recombinant virus, grown to monolayer, and transplanted into nude mice. This grafted tissue will serve as our in vivo model for mustard experiments.

Also immunohistochemical staining techniques for visualizing the enzyme and poly(ADP-ribose) polymerase have been perfected using the stably transfected keratinocyte cell lines (see below). This should allow us to localize enzyme and polymer within cells and determine the effect of vector-introduced exogenous enzyme. Such experiments will also define for us the optimum conditions for in vivo experiments with grafted skin, so that little time will be wasted in generating reliable in vivo data.

Currently we are collaborating with Dr. Takashi Shimadu and Dr. Arthur Nienhaus at the National Institutes of Health to create high-titer viral-producing cell lines as a perpetual source of recombinant virus for in vitro and in vivo experimentation. Using the recombinant DOL constructs produced in our laboratory and the retroviral packaging cell lines they hold, we hope to generate enough recombinant clones to fulfill our needs.

Our strategy is to transfect the plasmid vectors into a primary packaging line, and allow time for transient expression of the proviral cDNA-recombinant RNA. The RNA is packaged into intracellular viral particles that are constitutively produced within the cell line. The packaged RNA particles are shed into the medium from which they are recovered. The particles are then used to infect a secondary packaging line. Within the second packaging line the RNA message is reverse transcribed and the DNA transcripts become stably integrated into the cell genome as provirus. A murine leukemia virus LTR is used as a promoter for the recombinant virus, and this allows for a high rate of viral transcript production within the cells. Virus is recovered by centrifugation of the cell medium. The virus is titered by serial dilution and infection of the intended host cells. Infected cells are assayed based on virally-induced G418 resistance.

Drs. Shimadu and Nienhuis are experts in the field of retrovirally-based vectors, and we anticipate generation of useful producer cell lines presently.

#### Midterm Results on Immunological Methods

The complete amino acid sequence of poly(ADP-Ribose) polymerase, which my group published during the midterm, has allowed us to systematically assign three peptides to be synthesized which would allow us to produce large quantities of additional polyclonal and monoclonal antibody to the enzyme for all the studies that will be required for the contract period. It should be noted that the cDNA sequencing was performed with non-USAMRICD funds. The three peptides chosen from the sequence are assigned the names A, B, and C.

The A peptide is derived from the amino-terminus of the polymerase enzyme and will induce antibodies reactive to the DNA-binding domain of the protein. The antibodies elicited by peptide B correspond to the region of the enzyme which undergoes automodification, while the antibodies elicited from the use of peptide correspond to the active site of the polymerase enzyme. These peptides were sent to a colleague in Australia for injection into mice and monoclonal antibody production. A number of positive clones have been obtained and ELISA performed on culture supernatants. The results suggest that active monoclonal antibody has been obtained. We have recently received the supernatants. We are presently evaluating these by : (1) Western blot analysis of polymerase; (2) ELISA; (3) immunoprecipitation of polymerase; (4) immunostaining of cells after mustard treatment.

In addition, we are attempting to express large quantities of cloned polymerase in *E. coli* or yeast in order to provide more needed antigen for antibody, to supplement existing polyclonal antibody.

### Results on Immunostaining Techniques at Midterm

Our goal of identifying the mechanism of poly(ADP-ribose) polymerase involvement in DNA damage caused by mustards relies on the use of immunological staining methods to visualize ADP-ribose polymer in human skin tissue sections. For this reason, we have determined the parameters for optimal cellular staining using human cells in tissue culture.

Sugimura and co-workers (5) have reported staining of ADP-ribose polymer in rat cells with immunofluorescence techniques. Furthermore, they reported that staining could be enhanced by incubating the cells with NAD-- the substrate for the polymerase presumably due to a persistence of enzyme activity in the fixed cells. We sought to evaluate this technique for use in human cells in our laboratory.

Two human cell lines have been used. One was a human skin tumor cell line (melanoma) and the other was a human epithelial tumor cell line (HeLa). Both of these lines are relevant to the human skin situation. The cells were grown on coverslips, fixed, and incubated with and without NAD. Following the NAD treatment, the cells were incubated with antibody against ADP-ribose polymer. This was followed by incubation with a secondary fluorescently-labelled antibody. The cells were then examined by microscopy.

The results showed that NAD influenced fluorescence produced by anti-polymer antibody binding. This confirms the utility of this technique for studies of ADP-ribose polymer metabolism in the human skin system.

### CONCLUSIONS

(1) First, the immunology required for the project has been established in the laboratory. We have worked out methods to detect the generation of poly ADP-ribose in tissue culture cells by immunofluorescence. We are in the process of beginning to assess the influence of DNA strand breaks, as generated by sulfur mustards, on poly(ADP-ribose) content of cells. The model to be ultimately tested is that reduced NAD levels in skin cells and tissues contributes to the blistering effects noted in these agents.

Additionally, good progress has been made in producing large amounts of monoclonal antibody to the polymerase enzyme peptides?). We anticipate utilizing these for both immunostaining and immuno-precipitation studies during the second period.

The major prospects for the future of the project revolve around the possibilities for engineering human keratinocytes to overproduce or underproduce cloned poly(ADP-ribose) polymerase. Potentially, this may render a cell with greater resistance to mustards (and perhaps other agents). We also hope to eventually transplant such keratinocytes, as a layer, on mouse skin for *in vivo* studies. We have approached this by constructing the polymerase cDNA into retroviral expression vectors. The results of this approach so far, shown above, show that we have successfully constructed three key clones in the DOL retroviral vector: (1) the sense orientation of polymerase; (2) the antisense; (3) the 5' DNA-binding fragment [the latter may, when expressed, produce an active inhibitor of the enzyme, *in vivo*]. These constructs are all in the process of being tested.

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